



ADDENDUM



## Microbial and metabolic impacts of trehalose and trehalose analogues

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### ABSTRACT

Trehalose is a disaccharide and fasting-mimetic that has been both canonized and vilified for its putative cardiometabolic and microbial effects. Trehalose analogues are currently under development to extend the key metabolic therapeutic actions of trehalose without adversely affecting host microbial communities. In the current study, we contrast the extent to which trehalose and its degradation-resistant analogue, lactotrehalose (LT), modulate microbial communities and host transcriptomic profiles. We demonstrate that trehalose and LT each exert adaptive metabolic and microbial effects that both overlap and diverge. We postulate that these effects depend both upon compound stability and bioavailability, and on stereospecific signal transduction. In context, the data suggest that trehalose is unlikely to be harmful, and yet it harbors unique effects that are not yet fully replicated by its analogues. These compounds are thus valuable probes to better define trehalose structure-function, and to offer as therapeutic metabolic agents.

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## Introduction

In the past generation, multiple events have converged in the fields of microbiology, industry and medicine, which now enable us to consider a new, autophagy-promoting therapeutic agent to prevent or treat metabolic disorders. In 1995, low-cost, large-scale production processes were developed for trehalose, a now ubiquitously used disaccharide that is comprised of two glucose moieties linked by a 1,1- $\alpha$ , $\alpha$ -glycosidic bond. In addition to its industrial and commercial uses, trehalose was defined as a fasting-mimetic, mTOR-independent, autophagy-inducing agent.<sup>1</sup> Numerous applications of this agent in neurodegenerative, retinal, renal, infectious, and metabolic disease were reported quickly thereafter.<sup>2–15</sup> Advances in trehalose manufacturing technology were quickly followed by regulatory approval for its use as a food additive in Japan (1995), the United States and Europe (2000 and 2001), and in Canada (2005). This was a boon for biomedical, industrial, and commercial sectors, as trehalose is now used as a drug excipient, packing material, humectant, common foodstuff, and low glycemic-index sweetener.<sup>16</sup> Simultaneously, *Clostridioides difficile* (CD) ribotypes 027 and 078 emerged as causes of epidemics

and serious human disease. Several reports proposed a link between the emergence of these strains and the promulgation of trehalose. Specifically, (i.) there was temporal overlap between these two events, and (ii.) 027 and 078 possess trehalose catabolizing pathways that confer CD027 and CD 028 growth advantages in the absence of glucose.<sup>17,18</sup> These observations raised the hypothesis that trehalose directly contributed to the emergence and virulence of these epidemic CD strains.<sup>17,18</sup> The current work examines the evidence in regard to this hypothesis, and extends our recent work characterizing the microbial and metabolic effects of trehalose and its analogues.

## The evolution of trehalose-catabolizing CD ribotypes

CD infection is a leading cause of nosocomial antibiotic-associated diarrhea in industrialized countries, and is a major threat to human health.<sup>19</sup> Clinical manifestations of CD infection include diarrhea, pseudomembranous colitis, and in extreme cases, death. Putatively hypervirulent CD ribotypes 027 and 078 were first reported in 1985 and 1991, respectively, whereas a third CD ribotype, 017 was recently identified.<sup>17</sup> Each of these strains evolved

the ability to catabolize trehalose to fuel growth in glucose-limiting conditions.<sup>17,18</sup> Mechanisms by which these variants utilize trehalose include hypersensitive de-repression of the phosphotrehalase, *TreA* gene, and more efficient trehalose transport from the extracellular space via the PtsT transporter.<sup>17,18</sup> Enhanced enzymatic phosphotrehalase activity permits these variants to cleave trehalose into its component glucose monomers, which are then used as fuel for growth. *In vitro*, these variants grow equivalently in the presence of glucose, whereas CD 027, 078 and 017 also grow nearly unabated in the absence of glucose, as long as trehalose is provided as substrate.<sup>18,20,21</sup> The rise of trehalose manufacture and consumption was associated with the appearance of these trehalose-metabolizing ribotypes, which generated the hypothesis that trehalose contributed to the emergence and virulence of these ribotypes worldwide.<sup>17,18</sup> Sequential reports documenting these adaptations drew significant attention across scientific and lay communities.<sup>22-25</sup>

### Role of trehalose in CD 017, 027 and 078 emergence

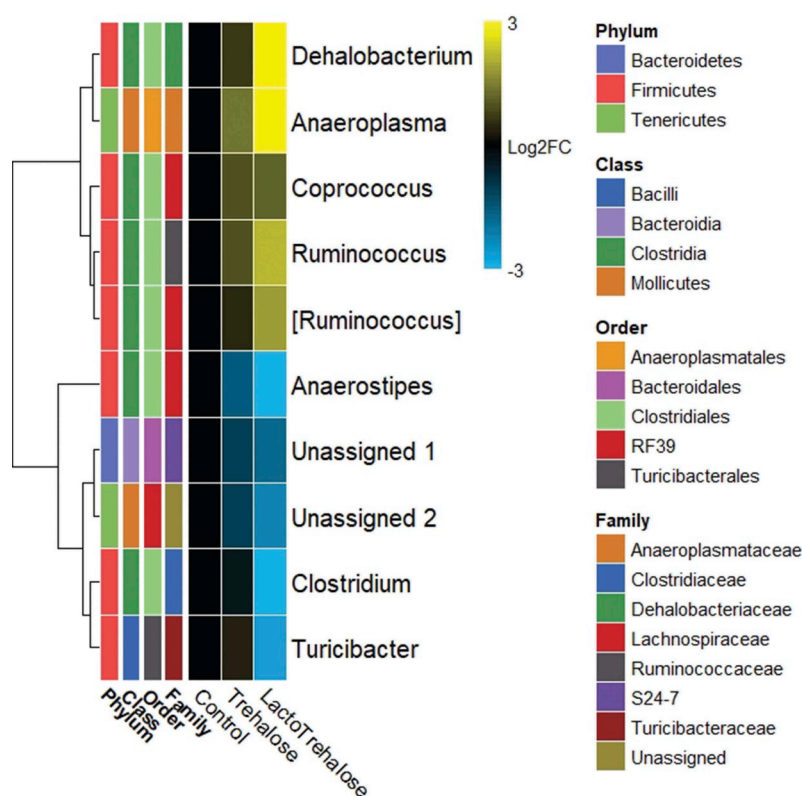
Recent speculation that dietary trehalose drove the emergence and virulence of CD 017, 027 and 078<sup>18,20</sup> did not fully account for several key pieces of data. First, *in vivo* colonic luminal glucose concentrations are generally high, and they exceed the zero-glucose concentrations that are used to model *in vitro* CD growth.<sup>26,27</sup> Because wild-type and trehalose-metabolizing strains grow equivalently in the presence of glucose, a proliferative advantage for trehalose-metabolizing variants in the *in vivo* colonic environment is not expected. Second, trehalose-metabolizing CD variants are not clearly more virulent in humans than other CD ribotypes.<sup>28-30</sup> Accordingly, metabolomic and microbial analyses in patients demonstrate no correlation between trehalose content in the colonic metabolome with CD infection.<sup>31</sup> Third, trehalose metabolic CD variants are common, and yet only a subset of these variants successfully emerged, even if originating from within the same clade as other trehalose-metabolizing strains.<sup>28</sup> This selectivity occurred despite widening use of trehalose in industrial and commercial applications,<sup>28</sup> which indicates that enhanced trehalose metabolic capacity *per se* is not sufficient to

modulate CD variant growth and virulence. Fourth, CD 027 infection stabilized or even declined in the US and EU after 2006, despite continued increases in trehalose distribution worldwide.<sup>28,32</sup> In particular, CD 027 cases in the US dropped precipitously after 2010, although the etiology of this observation is not yet clear.<sup>32</sup> Finally, trehalose does not increase CD proliferation or total abundance in two distinct *in vivo* CD 027 infection models,<sup>11,18</sup> and does not exacerbate CD 027-induced rectal tissue inflammation, rectal or fecal inflammatory marker gene expression, or CD toxin A or B abundance.<sup>11</sup> Together, current epidemiologic, clinical, physiological and animal modeling data do not substantiate recommendations against dietary trehalose consumption as a means to protect against CD emergence and virulence.

### Microbial impact of altering trehalose carbon-4 stereochemistry

Whether or not trehalose played a central, peripheral, or negligible role in the emergence and virulence of specific CD ribotypes, degradation-resistant trehalose derivatives have emerged as equally promising therapeutic agents.<sup>11,12,33-36</sup> These analogues, including 5-thiotrehalose, mannotrehalose, and lactotrehalose (LT), overcome at least two major barriers to translating trehalose to the bedside as a therapeutic agent. First, degradation-resistant trehalose analogues do not promote CD 027 or 078 growth *in vitro*<sup>33</sup> or in a mouse model of CD 027 infection.<sup>11</sup> Second, these analogues resist cleavage by microbial and host brush-border trehalases,<sup>11,33,36</sup> which increases their bioavailability to organs outside the portal venous circulation.<sup>12</sup> This latter characteristic is expected to render these analogues particularly useful to treat extrahepatic diseases that are amenable to trehalose treatment.

LT differs from trehalose in its hydroxyl group orientation at carbon 4. This alteration confers resistance to degradation by trehalases.<sup>21</sup> Accordingly, LT has dominant inhibitory effects on CD 078 and CD 027 proliferation in both glucose-limiting and glucose-replete conditions.<sup>33</sup> Moreover, neither LT nor trehalose exacerbates CD 027-induced rectal inflammation or CD Tox A or Tox B production in mice.<sup>12</sup> In light of these specific effects of trehalose analogues on CD and on mycobacterial species in prior



**Figure 1.** Heatmap demonstrating mean  $\text{Log}_2(\text{fold change})$  for significantly altered bacterial taxa (adjusted  $P < .05$ ), as determined by 16 S ribosomal DNA sequencing in stool obtained from mice fed vehicle, trehalose or LT (3% disaccharide in drinking water, *ad libitum*, 5 days).

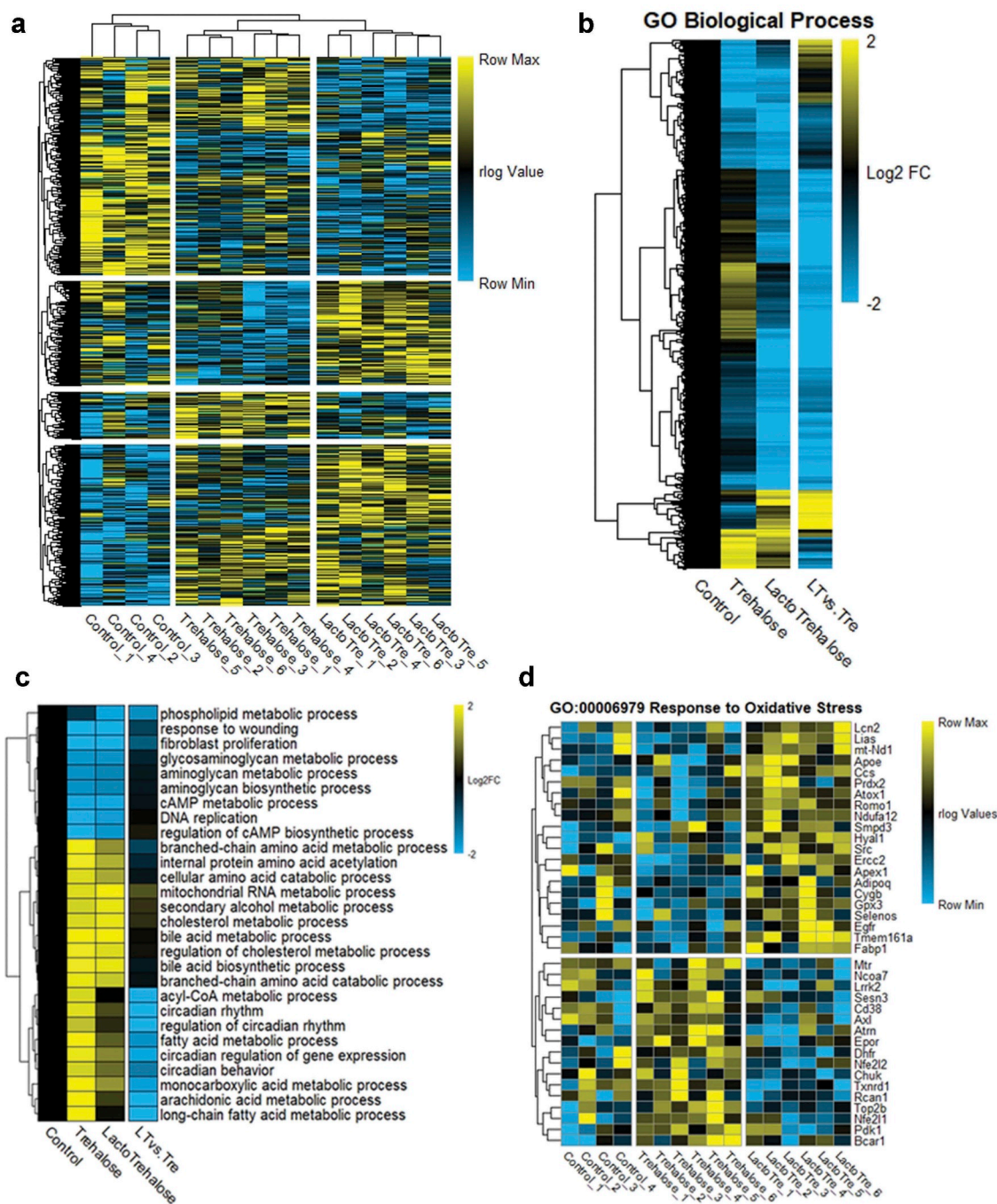
work,<sup>14,35,37</sup> we examined the broader microbial effects of trehalose and LT by 16 S ribosomal DNA sequence analysis in stool obtained from mice treated with vehicle, trehalose, or LT (Figure 1). LT produced more pronounced shifts in Bray-Curtis dissimilarity along two principal components at the operational taxonomic unit (OTU) level than did trehalose.<sup>12</sup> *Dehalobacterium* and *Ruminococcus* were enriched in LT-treated mice, compared to vehicle-treated mice, whereas *Turicibacter* decreased in LT-treated mice (Figure 1). These fasting-mimetic microbial effects mirror the changes observed in fasting *db/db* diabetic mice<sup>38</sup> and in fasting lower vertebrates.<sup>39</sup>

### Metabolic impact of altering trehalose carbon-4 stereochemistry

Apart from its microbial effects, LT enhances hepatocyte autophagic flux and FGF21 protein abundance *in vitro*.<sup>12,34</sup> In mice, lactotrehalose bioavailability is greater than that of trehalose, and it increases basal heat production,  $\text{VO}_2$  and liver mitochondrial oxidative metabolism to a greater

extent than trehalose.<sup>12</sup> Yet, the metabolic effects of LT may extend beyond trehalase insensitivity and enhanced bioavailability. Specifically, beta-diversity principal component analysis from 16 S rDNA sequencing suggests that LT generally induces analogous, but more extreme colonic microbial and microbial metabolic gene expression shifts (inferred from bacterial ORF analysis) when compared with trehalose.<sup>12</sup> In contrast, liver transcriptomic data in LT- and trehalose-treated mice reveal surprisingly divergent transcriptomic effects. With regard to host metabolic effects, LT does not behave simply as a more potent or more bioavailable form of trehalose. Rather, trehalose and LT each induce both overlapping and unique, metabolically consequential genes (Figure 2(a)) and gene ontology (GO) pathways (Figure 2(b)). For example, GO analysis previously revealed that the top LT-induced pathways were mitochondrial gene expression, oxidative phosphorylation, and electron transport chain pathways, to a greater extent than trehalose.<sup>12</sup> These transcriptomic data were validated by physiological data demonstrating increased mitochondrial oxidative





**Figure 2.** Divergent hepatic transcriptomic effects of trehalose and LT. (a). Heatmap demonstrating individual liver gene expression patterns and selected Gene Ontology (GO) pathways (right) that are significantly altered when comparing trehalose vs. lactotrehalose in mice fed vehicle, trehalose, or LT (3% disaccharide in drinking water, *ad libitum*, 5 days). Left-to-right, relationships mapped are: trehalose versus control; LT versus control; LT versus control.  $P < .05$  for all LT versus trehalose relationships demonstrated. (b and c). GO pathway analysis demonstrating significantly different pathways (b). and demonstrating significantly different, specific metabolic GO pathways (c). from bulk RNAseq data derived from livers analyzed in (a). The pathways shown are significantly different when comparing trehalose and LT treatment groups. (d). Heatmap of transcripts in the oxidative stress response GO pathway. The pathway overall does not differ when comparing mice treated with trehalose or LT (3% disaccharide in drinking water, *ad libitum*, 5 days). Demonstrated here are the individual genes within this pathway that are significantly altered when comparing livers from vehicle-fed mice vs. trehalose-treated or vs. lactotrehalose-treated mice.

phosphorylation by Oroboros respirometry.<sup>12</sup> In contrast, trehalose increases genes in the circadian rhythm pathway, and the metabolism of fatty acids,

and of long-chain fatty acids, arachidonic acid, monocarboxylic acids, thioesters, and glycogen metabolism to a significantly greater magnitude

than LT (Figure 2(c)). Several of these trehalose-induced gene pathway effects were also validated by *in vivo* mouse genetic methods.<sup>5,11,34</sup>

Exemplifying key distinctions between trehalose and LT at the gene-specific level, trehalose induced antioxidant genes<sup>40-42</sup> topoisomerase 2b (*Top2b*) and thioredoxin reductase (*Txnrd1*), to a greater extent than LT (Figure 2(d)). By comparison, LT uniquely induced lipocalin (*Lcn2*) and lipoic acid synthetase (*Lias*), each of these which is important in the hepatoprotective anti-oxidant response, and each of which is uniquely regulated by trehalose and LT.<sup>43-45</sup> Moreover, we demonstrated previously that LT more potently induces canonical trehalose effectors, hepatocyte *Pgc1α*, *Fgf21*,<sup>46</sup> *Aloxe3* and *Arg2*.<sup>34,47</sup> Differential transcriptomic responses highlight divergent metabolic effects of each disaccharide. This opposes a model in which LT acts as a higher-amplitude trehalose-mimetic stimulus. Therefore, to ascribe the mechanistic differences between these analogues solely to differences in their trehalase susceptibility and bioavailability is probably too simplistic.

We hypothesize that part of the bioavailability-independent effects of trehalose and its analogues are accounted by signaling events via specific interactions with the carbohydrate transporters,<sup>4,5</sup> or via downstream intracellular signaling pathways with which these sugars interact.<sup>5,6,13</sup> For example, although both LT and trehalose have comparable GLUT8 inhibitory capacity, trehalose is a more potent inhibitor against other class I GLUTs.<sup>12</sup> In addition, trehalose activates TFEB and FGF21<sup>7,13,46,48,49</sup> through yet-incompletely defined mechanisms. Forthcoming work should directly examine structure-function relationships and specific mechanistic actions for these compounds.

## Concluding remarks and future perspectives

The technological advances that brought trehalose into our industrial, commercial, and biomedical environments over the past two decades highlighted both its promising utility, and its potential barriers to address. At present, the potential microbial<sup>14,35,</sup> and metabolic effects of trehalose is weighed against potential CD growth-promoting effects. At least two major questions thus remain before we can optimally use trehalose and its analogues as therapeutic agents.

First, what is the basis for the observed metabolic differences in trehalose and trehalose analogue action, and how is trehalose best manipulated to capture the breadth of adaptive metabolic and microbial effects? Second, did trehalose drive the emergence and virulence of trehalose-metabolizing CD ribotypes? Current epidemiological, clinical, and murine modeling data do not favor a deleterious effect of trehalose. Yet, a rigorous answer to this question is critical, because native trehalose exerts specific metabolic effects which we do not yet understand, and which are not yet replicated in degradation-resistant trehalose analogues. Trehalose thus remains a viable industrial, commercial and biomedical therapeutic option to examine and carry forward. Convergent work across metabolic, biochemical, and microbial disciplines will elucidate these important questions, advance exploration of native trehalose structure-function, and develop new trehalose analogues that retain and extend trehalose's full effects without adversely impacting host microbial communities.

## Original article information

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## Disclosure of Potential Conflicts of Interest

BJD Receives reagent-grade trehalose and LT from Hayashibara Co, Japan, which manufactures trehalose.

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## Author contributions

Designed the studies: YZ, BJD. Conducted experiments: YZ, BJD. Analyzed the data: YZ, BJD. Wrote the manuscript: BJD and YZ. All co-authors approved the manuscript.

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